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	Patent ADP number *(if you know it)*	7125487005
	If the applicant is a corporate body, give the country/state of its incorporation	SWITZERLAND
4.	Title of invention	Organic compounds
5.	Name of your agent *(If you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	~~B.A. YORKE & CO. CHARTERED PATENT AGENTS COOMB HOUSE, 7 ST. JOHN'S ROAD ISLEWORTH MIDDLESEX TW7 6NH~~ Craig McLean Novartis Pharmaceuticals UK Ltd. Patents + Trademark Wimblehurst Rd Horsham West Sussex RH12 5AB
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Organic Compounds

The present invention relates to organic compounds, e.g. to an assay for identifying an agent that modulates the activity of a CD45 tyrosine phosphatase.

In conventional ELISA systems in general either the substance, e.g. an antigen which is to be detected or a specific antibody which binds to the respective antigen, is bound to a solid phase (e.g. microtiter plate) by hydrophobic interactions: the protein interacts with the solid phase, usually a polystyrene surface, at high pH. Although this bond is responsible for all the consecutive steps in the procedure, it remains the weakest bridge to the assay support, the ELISA plate. Strong detergents at higher concentrations such as 0.05% are able to diminish the amount of reagent bound to the plate and may even abolish binding totally. Consecutive steps in ELISA technique, such as e.g. the binding of an antigen to the solid phase-bound antibody and further binding of a second antibody, occur with an affinity of approximately 10^{-12} to 10^{-10} mol per liter. One can view this as being similar to an inverse binding cascade from the bottom of the plate to the top, comparable with a pyramide standing on the top. Another concern is the intramolecular event upon binding. A protein such as a cell-receptor, an enzyme or an antibody behaves very flexible according to its polypeptide structure, which forms a complex architecture in solution. This explains its high specificity and selectivity to the ligands to which they bind in vivo. Enzymatic activities for example may be entirely dependent upon the proper formation of the active site pocket, which itself remains flexible in order to engulf the substrate and release the product.
Most proteins when binding to a given surface react with a dramatic change of their tertiary structure, i.e. they unfold, refold, hide their active site or change their conformation in such a way that their activity towards a given ligand is altered or even abrogated. In order to circumvent this disadvantage, in conventional ELISA systems a catching antibody is generally used. This antibody binds to the polystyrene plate and exposes the high affinity hyper-variable region towards the incomig antigen. The antigen is then detected by a second antibody, which is labelled directly or indirectly (e.g. via biotin/avidin) with an enzyme. This enzyme is able to cleave a chromogenic substrate, which itself is converted from the leucoform to the chromoform and thus visualizes the presence of the antigen in question. But even catching antibodies may affect a given protein and its conformation may be changed. This is demonstrated by many examples of therapeutic antibodies whose mode of action is the blocking of an active site on, or the alteration of a biospecific molecule.

We have now found that in using an assay according to the present invention structural influences or sterical hindrance by the solid phase may be avoided and that since all reaction partners are present in defined molar concentration determination of reaction products may be highly accurate and specific.

In one aspect the present invention provides a method for identifying an agent that modulates the activity of a CD45 tyrosine phosphatase comprising:

a) providing a phosphotyrosine containing peptide, preferably a labeled peptide, e.g. a biotinylated peptide,
b) providing a CD45 tyrosine phosphatase,
c) contacting the peptide of a) with the CD45 tyrosine phosphatase of b) in the absence and in the presence of a candidate compound which is expected to modulate the activity of a CD45 tyrosine phosphatase for a sufficient period of time so that a reaction mixture is formed and reacting for a pre-determined period of time,
d) transferring at least an aliquot of the reaction mixture formed in c) to a solid phase, preferably a solid phase coated with a substrate which is able to bind to the label of the peptide, e.g. a streptavidin-coated solid phase for a biotinylated peptide,
e) detecting the amount of phosphotyrosine moiety of the peptide bound to the solid phase of d) and determining whether there is a difference in the amount of phosphotyrosine in case a candidate compound was present or absent in the reaction mixture, and
f) choosing an agent from said candidate compound detected in e), e.g. for use as a pharmaceutical.

The principle of this method or assay is as follows: One of the characteristics of a CD45 tyrosine phosphatase is that this enzyme is able to dephosphorylate a phosphorylated substrate, e.g. a phosphorylated tyrosine (= phosphotyrosine) containing peptide. The substrate may be provided in a labeled form, e.g. as a biotinylated peptide. After a defined contact/incubation time of the phosphatase and the substrate, e.g. a peptide according to a), under defined reaction conditions, like e.g. controlled temperature, pH, salt concentration etc., the reaction is stopped and an aliquot of the reaction mixture is transferred to a solid phase which is preferably coated with a substrate able to bind to the label of the peptide, e.g. a streptavidin-coated solid phase like e.g. a streptavidin-coated microtiter plate for detecting e.g. a biotinylated phosphotyrosine containing peptide. That peptide is thereby trapped and may be quantified e.g. by means of an appropriate enzyme or fluorescence-labeled

antibody, against the phosporylated peptide or by means of a second antibody specifically recognising the first antibody and bearing a label, e.g. an enzyme or fluorescence label.

All the components used in the method or in an assay, e.g. the peptide of a), the tyrosine phosphatase of b) and optionally also the antibody, are preferably provided in solution wherein the concentration of each component is known. Therefore, during the whole reaction all components involved are free in solution and are thus not influenced by the structural modifications of e.g. solid phases. They receive their conformation by the given pH and salt concentration and exert their maximum binding or reaction affinity. Also the exact amounts of all the reaction components are known. After a given contact/incubation time, selected according to the results of appropriate equilibrium measurements, the peptide is deprived of all other components. The peptide is preferably pre-labeled, e.g. biotinylated. An aliquot of the reaction mixture is transferred to a solid phase, e.g. a streptavidin-coated microtiter plate, and the phosphotyrosin moiety of the peptide is detected.
The solid phase is preferably a plastic plate like a polystyrene or polyvinyl plate, esp. a microtiter plate. Also microbeads may be used as a solid phase, preferably coated microbeads. The coating used for the solid phase depends e.g. on the label used for the peptide. The material of the coating should be able to form a complex with the label used for the peptide, e.g. the material used for the coating may be streptavidin and the label used for the peptide may be biotin.

In case a candidate compound has a modulating, e.g. inhibiting, effect on the CD45 tyrosine phosphatase, the phosphatase has no or a limited activity to dephosphorylate the peptide. Therefore all or a smaller amount of phosphotyrosine of the bound peptide will be detected. In comparing the amounts of remaining phosphotyrosine in the presence and in the absence of a candidate compound an appropriate agent may be identified. Since the reaction takes place in solution at well-defined molar concentrations of all the essential components used in this assay, and at defined temperature, pH and salt conditions, structural influence of solid phases are perferctly eliminated in this assay.

A candidate compound includes compound(s)(libraries) from which its influence on the CD45 tyrosine can be determined. Compound (libraries) include for example oligopeptides, polypeptides, proteins, antibodies, mimetics, small molecules, e.g. low molecular weight compounds (LMW`s).

An agent is a compound which influences (inhibits) the expression of the reporter gene and/or its products detected/determined in c).
An agent is one of the chosen candidate compounds and may include oligopeptides, polypeptides, proteins, antibodies, mimetics, small molecules, e.g. low molecular weight compounds (LMW's). An agent includes one or more agents.

The phosphotyrosine containing peptide comprises a sequence with specificity for a tyrosine phosphatase. Preferably the peptide comprises a sequence selected from the group consisting of T-E-G-Q-Y-Q-P-Q-P, R-N-Q-E-T-Y-E-T-L-K-H and A-E-N-T-I-T-Y-S-L-L-M-H-P wherein Y is phosphorylated.

The amount of phosphotyrosine can be detected with a labeled anti-phosphotyrosine antibody or in using a tandem system of first anti-phosphotyrosine antibody and a second antibody specificall recognising the first antibody and bearing a label, e,g, an enzyme or fluorescence label.

In a preferred embodiment the binding of e.g. streptavidin with the plate surface is perfectly made covalent in order to build up an affinity cascade from the bottom of the microtiter plate to the top of the reactants. For example, a microtiter plate chemically modified with e.g. a coating with an N-oxysuccinimide ester is used, which reacts with nucleophiles such as primary amines under formation of a covalent bond. This bond is stronger than 10^{-15} moles per liter. For the next step, the labeling, e.g. biotinylation of the peptide, e.g. a chemically modified biotin, such as NHS-LC-Biotin is used, which comprises an extended spacer arm of approximately 22.4 Å in length. This long chain analogue reduces steric hindrance associated with the binding of four biotinylated molecules on one streptavidin molecule. The target of biotinylation on the peptide is thereby well defined and interference with the ligand is avoided. The binding affinity of e.g. biotin to streptavidin is also known to be 10^{-15} moles per liter. Thereby the first two steps of the binding cascade are established and reach from the bottom of the plate to streptavidin to the biotinylated peptide. The affinity constants are decreasing from the bottom to the top. At this stage the reactants, which have found their partners in solution under defined conditions, are trapped by means of the streptavidin plate, and the phosphotyrosine moiety of the bound peptide is then detected with an appropriate detection system as described above.

Some of the advantages of the assay according to the present invention in comparison to standard ELISA systems where the phosphatase or the peptide will first be attached to the solid support with e.g. either a catching antibody or by high pH (9.6) are the following:

a) The structural influence or sterical hindrance by the supporting solid phase or the catching antibody in standard sandwich-ELISA is avoided,

b) no treatment of the phosphatase or the peptide at alkaline pH,

c) the reaction partners are present in defined molar concentrations, and

d) reduced working steps and easy handling by e.g. robotics.

In another aspect the present invention provides a kit for identifying an agent that modulates the activity of the CD45 tyrosine-phosphate phosphatase comprising as components

a) a phosphotyrosine containing peptide, preferably a labeled peptide, e.g. a biotinylated peptide,

b) a CD45 tyrosine phosphatase,

c) an anti-phosphotyrosine antibody, and

d) optionally a solid phase, preferably coated with a substrate which is able to bind to the label of the peptide.

Said kit may further comprise a substantial component including e.g. an appropriate environment of a sample to be tested and, e.g. appropriate means to determine the effect of a candidate compound in a sample to be tested.

The peptide comprises preferably a sequence selected from the group consisting of T-E-G-Q-Y-Q-P-Q-P, R-N-Q-E-T-Y-E-T-L-K-H and A-E-N-T-I-T-Y-S-L-L-M-H-P wherein Y is phosphorylated.

In another aspect the present invention provides the use of a peptide comprises a sequence selected from the group consisting of T-E-G-Q-Y-Q-P-Q-P, R-N-Q-E-T-Y-E-T-L-K-H and A-E-N-T-I-T-Y-S-L-L-M-H-P wherein Y is phosphorylated for identifying an agent that modulates the activity of a CD45 tyrosine phophatase.

In another aspect the present invention provides a method for identifying an agent that modulates the activity of a CD45 tyrosine phophatase comprising an assay according to the present invention.

In the following examples all temperatures are in degree centigrade and are uncorrected.

The following ABBREVIATIONS are used:

BSA	bovine serum albumin
DTT	dithiothreitol
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FPLC	forced pressure liquid chromatography
IPTG	isopropyl-β D-thiogalactopyranosid
LC	long chain spacer arm
mAb	monoclonal antibody
MW	molecular weight
PBS	phosphate-buffered saline
PBSdef.	NaCl 8 g/l; KH_2PO_4 0.2 g/l; KCl 0.2 g/l; Na_2HPO_4.2aq 1.44 g/l; pH 7.2
PBST	PBS + 0.05% Tween 20
POD	horse radish peroxidase
pNPP	p-nitrophenylphosphate
PTPase	phosphotyrosine phosphatase
rpm	rounds per minute
RT	room temperature
TFA	trifluoracetic acid
TMB	teramethylbentidine
wwt	wt weight

EXAMPLES:

Example 1:

a) Preparation of LCA D1D2 (CD45) Phosphatase

The pT7 LCA D1D2 plasmid encodes both the D1 and D2 cytoplasmic domains of the human LCA PTPase. The LCA-D1D2 PTPase contains 8 amino acids (M-A-R-I-R-A-R-G) derived from the pT7-7 vector at the N terminus and residues 584 through the most C-terminal residue (position 1281) of the human LCA protein. (see e.g. J.Biol.Chem.Vol.267, pp12356-12363).

b) Overproduction of LCA-D1D2-PTPase

In order to overproduce this enzyme E.coli BL21 is transformed with the plasmid pT7-LCA-D1D2, which carries an integrated copy of the T7 RNA polymerase gene under the control of the inducible lacUV5 promoter. The transformed E.coli BL21 are assayed for the presence of the plasmid (mini-preps) and for the expression of the respective protein.

Stocks are prepared from the mid-log-phase by freezing the bacterial suspension in 50% glycerol at -190° and stored at -80°.

c) Purification of the LCA D1D2 PTPase

A 1 ml aliquote from the stock-culture is thawed and serves as inocculum for a 200 ml LB AMP 100 [500 ml Erlenmeyer flask]. The culture is agitated at 200 rpm and 37° to an E_{600} = 0.7, where 8 times 33 ml are transferred into 8 times 1 liter LB Amp 100. This culture is grown under the same conditions until an E_{600} of 0.7 is achieved. At this point 10 ml of a sterile stock solution of 100 mM IPTG is added in order to adjust the bacterial culture to 1 mM IPTG. The temperature is lowered to 28° and the agitation at 200 rpm is continued for three more hours. Cells are harvested by centrifugation at 18.200 x g at 4°. The cell mass is resuspended in 30 ml buffer A (33 mM Tris-HCl pH 8; 25 mM EDTA; 10 mM 2-mercapto-ethanol) to give a concentration of approx. 130 mg wwt/ml. In order to provide a rapid heat transfer the suspension is divided into 30 ml aliquotes kept in 50 ml vials.

The vials are submersed into liquid nitrogen until equilibrium is reached and afterwards they are submersed into a 37° waterbath until the content is liquified.

Then 2 ml of a 6 mg/ml solution of lysozyme (Boehringer) are added and the vials are agitated on a reciprocate shaker at 50 rpm at RT. Thereafter 1 ml Trasylol (Bayer), 30 µl Leupeptin (Boehringer Mannheim, 50 mg/ml), 2 ml 500 mM $MgCl_2$ a few grains of DNAse I

(Boehringer) are added to each vial, which are agitated as described above. The reaction is stopped by the addition of 2 ml 500 mM EDTA, 300 µl Triton-X100 are added and the solubilization procedure is carried on during agitation. The extract is clarified by centrifugation at 120.000 x g at 4°. All supernatants are pooled and adjusted to 35% ammoniumsulfate under slow addition of the powder under stirring at RT and subsequent stirring at 4°. The precipitate is collected by centrifugation as above and the supernatant is adjusted for 60% ammoniumsulfate under conditions mentioned before.

The precipitate containing the product is harvested by centrifugation as described above, where the pellet is dissolved by 10 ml buffer B (= 33 mM Tris-HCl pH 8.5; 2.5 mM EDTA; 10 mM 2-mercaptoethanol) plus 3 ml Trasylol and 0.5 ml Leupeptin. This sample is dialyzed at two consecutive steps at 4°: once against 2 liters of buffer B for one hour and then against 5 liters of buffer B.

The first chromatographic purification is done with a DEAE Sepharose Fast Flow column equilibrated in buffer B. All are carried out at 4°. The gradient is formed against buffer B containing 1 M NaCl. (flow rate 2 ml/min, gradient 0-30%: 0.1%/min; gradient 30-100%: 1%/min; sample application and wash: 12 ml/fraction; gradient: 4 ml/fraction).

The fractions are analyzed for phosphatase activity with p-nitrophenyl-phosphate and RNQETY(PO_3H_2)ETLKH as substrates (see h) assays). The fractions containing the specific phosphatase activity are pooled and concentrated by ultrafiltration (Amicon PM30/76) under addition of 2 ml Trasylol, 1 ml Leupeptin and 10 ml glycerol to approx. 20 ml. This sample is further purified over a Blue Sepharose column equilibrated in buffer B containing 10% glycerol; pH 7.4. The gradient is formed against buffer B containing 1 M NaCl; 10% glycerol; pH 9.4. (flow rate 2 ml/min, gradient 0-100%: 0.33%/min; sample application and wash: 12 ml/fraction; gradient: 4 ml/fraction).

The specific phosphatase containing fractions are pooled and concentrated by ultrafiltration (Amicon PM30/76) and addition of 10 ml Trasylol and 1 ml Leupeptin to 11 ml. This sample is loaded for consecutive runs at 2.5 ml onto a Superdex 75 size-exclusion column equilibrated in a buffer mixture of 40% buffer B in buffer A of above conditions. The chromatography is done at isocratic conditions and fractions of 2.6 ml are taken.

After assaying for phosphatase activity in both ways, the active fractions are pooled and concentrated to 12 ml. 1 ml of Leupeptin, 1 ml Trasylol and 46.5 ml of glycerol are added. This preparation is divided into 30 µl aliquotes which are stored in Micronic Racks (96 vials) at -80°. The final concentrations of such a preparation are the following:

Protein 728 µg/ml, Tris 8.9 mM, EDTA 0.7 mM, NaCl 108 mM, 2-Mercaptoethanol 2.7 mM, Aprotinin 32 µg/ml, Leupeptin 1124 µg/ml and glycerol 75%.
This preparation is assayed for activity using the gamma-substrate RNQETY(PO_3H_2)ETLKH as a serial dilution of the enzyme.

d) Biotinylation of peptide substrates

5 µmol of phosphotyrosine peptide is dissolved in 500 µl either DMF or DMSO and kept in an light protected vial. A solution of Immunopure NHS-LC-Biotin II (Pierce) is prepared in above solutes and kept under light protection. 500 µl of biotin solution is then added to the peptide containing vial, which will be tumbled at 25 rpm at RT. Thereafter another 500 µl of the biotin solution is added and the vial is tumbled for 3 hours under the same conditions. Then the reaction is stopped by addition of 500 µl of 1 M Tris-Cl pH 8 and tumbled for 30 minutes. The reaction mixture obtained is subsequently transferred into Eppendorf vials (500 µl each) and centrifuged 14.000 rpm in order to clarify the solution of biotinylated peptide. This supernatant is subjected to HPLC purification on Vydac C_{18} (218 TBP 10 µ 740711) equilibrated to 5% acetonitrile; 0.1% TFA. The peptide is eluted by a gradient toward 100% acetonitrile; 0.1% TFA. (flow rate 2 ml/min; gradient 0-100%: 1%/min, fraction size: 2 ml/fraction).

e) Preparation of covalently bound streptavidin microtiter plates

To 12 ml of PBSdef. of pH 9, 6 µl of streptavidin solution is added and stirred. 100 µl of this solution are pipetted into each well of a Costar Amine plate, which is taken directly from 4° storage and dismanteled from its protective envelope prior to pipetting. The whole procedure is performed in the dark and the plate must be kept in a light protective aluminium foil at RT. Thereafter the consecutive steps can be done under normal conditions:
Five times washing of the plate with PBST and tapping onto a paper towel in order to free the plate from residual moisture, addition of 360 µl of 0.5 M Tris-Cl pH 8 and incubation at RT. After a five times washing as above, 200 µl of blocking solution is added into each well and kept at RT for 30 minutes. Then the plate is washed once more and stored in a sealed plastic box at 4°.
The following peptide substrates are provided:

Lck	TEGQY(PO_3H_2)QPQP
Lck.biot	biotinyl-6-AhxTEGQY(PO_3H_2)QPQP
FcεR1γ	RNQETY(PO_3H_2)ETLKH

FcεR1γ.biot	biotinyl-6-AhxRNQETY(PO_3H_2)ETLKH
ITIM	AENTITY(PO_3H_2)SLLMHP
ITIM.biot	biotinyl-6-AhxAENTITY(PO_3H_2)SLLMHP

f) Assay for the detection of both biotinylation and function of the phosphotyrosine peptide

Into a U-bottom microtiter plate 50 µl of 200 mM $NaHCO_3$ is pipetted prior to 30 µl of fraction aliquotes in order to neutralize the TFA. 60 µl of this plate are copied into a microtiter plate covalent coupled with streptavidin, which is kept at RT. Thereafter the plate is washed 5 times with PBST and 100 µl of anti-phosphotyrosine antibody 1:2000 in incubation buffer is added to the plate. After incubation the plate is washed again as described above and 100 µl of anti-mouse-POD antibody 1:2000 in incubation buffer is added to the plate. After subsequent incubation the plate is washed with PBST as described above and 100 µl of TMB-substrate are added. After incubation at RT the reaction is stopped by the addition of 4 N H_2SO_4 and the plate is read at 450/690 nm. By this assay only those fractions give positive results which are tyrosine phosphorylated, not aggregated and properly biotinylated.

g) Cell-experiments

1. Cells (10^8/ml) are stimulated at 37° with F(ab')$_2$ (12 µg/ml) or intact anti-mIg (20 µg/ml). Unstimulated and stimulated cells are lysed in 1% NP-40 (Sigma) lysis buffer (= 1% NP-40; 10 mM Tris-Cl pH 7.4; 150 mM NaCl; 400 µM EDTA; 1.4 ml/liter Trasylol) and spun at 14.000 rpm in an Eppendorf microfuge to remove detergent-insoluble material.
A serial dilution of this cell lysate in assay-buffer (160 µl) is incubated with 80 µl of biotinylated peptide substrate (3 nM) at 37°. Thereafter a 100 µl aliquote is transferred to a Streptavidin-covalent plate and incubated at 4° (see e.g. Science,268,(1995), p293-297).

2. Cells (10^8/ml) are treated with inhibitors at different concentrations and different times of incubation. Thereafter 240 µl of the respective sample is pipetted into the top-row of a U-bottom microtiter plate A1-A12 where 60 µl of a 5-fold concentrated lysis buffer (see above) are already placed. There the cells are lysed by mixing with a 12-channel pipettor. 100 µl of the lysate are transferred into 200 µl of substrate at 1.5 nM concentration (row B1-B12), mixed and further diluted into substrate at 1 nM concentration (row C1-C12) by transferring 100 µl into 200 µl of substrate. These steps are consequently repeated from row C to row H. The plate was then incubated at 37° and thereafter stopped with 25 mM sodium-

meta-vanadate. 100 µl of the plate is copied into a streptavidin-microtiter-plate (SA-covalent, see SOP 21). The amount of phosphotyrosine is detected as described below.

h) Assays

1. Phosphatase assay during purification procedure for LCA D1D2

The assay buffer described here is identical with the system for peptide substrates, namely: 100 mM Tris, 5 mM EDTA, 10 mM DTT, 50 mM NaCl, 50 µg BSA/ml, 1.4 ml Trasylol/liter, 5% glycerol at pH 7.2. For column fraction assays this buffer system is adjusted to 10 mM p-nitrophenylphosphate.

20 µl fraction aliquotes are pipetted into a microtiter plate (flat bottom), 100 µl of substrate are added and the plate is incubated at RT. The reaction is stopped by addition of 100 µl 132 mM NaOH (60 mM final concentration) and the plate is read at 405 nm.

For specific assay for column fractions 10 µl of sample is placed into each well of a U-bottom microtiter plate and 40 µl of assay buffer are added. Thereafter 50 µl of the gamma substrate are added and the plate is incubated at 37°. 100 µl of aliquote are transferred to a streptavidin-plate and processed as described below.

2. Specific Phosphatase Assays

All substances are distributed into microtiter plates for testing in solutions of 10 mM NaCl; 50% DMSO at a concentration of 200 µg/ml or 500 µM. These solutions in conical bottom plates are diluted once more 1:3.3 (20 µl of substance plus 46 µl of assay buffer) into round bottom plates in order to give 66 µl 150 mM substance in 15% DMSO. These plates are adjusted in position A4-A12 by emptying the wells by suction (the whole line A1-A3 is dedicated for medium for medium references = High control) and 1 M HCl is pipetted into well A9-A12 in order to give the Low control. Sodium-vanadate at 500 mM is adjusted to pH 7.2 and diluted to 40, 20, 10, 5, 2.5 mM and pipetted into A4-A8 in order to give the calibration curve for sensitivity. This plate is assigned as dilution plate.

The working solution for the biotinylated peptide substrate is prepared by diluting the stock solution in assay buffer to a final concentration of 3 nM. 50 µl of this solution is distributed to each well of a new round bottom plate (incubation plate) and 50 µl of the respective dilution plate is transferred to this plate and mixed with the peptide substrate for five times. The plate is kept at RT and thereafter 50 µl of an adequate dilution of CD45 phosphatase in assay buffer (4.8 µl/ml) is added to each well of the incubation plate and mixed five times. The respective concentration are as follows:

5% DMSO, 50 µM substance, 300 mM HCl, 1nM peptide substrate, 13.3; 6.7; 3.3; 1.7; and 0.8 mM sodium vanadate.

The plate is sealed with an adhesive foil and incubated for one hour at 37°. The reaction is stopped by addition of 50 µl 25 mM sodium-meta-vanadate and 100 µl are transferred from the incubation plate into the streptavidin-coated plate, which is incubated at RT. After a five times wash with PBST 100 µl of a mixture of anti-tyrosine-phosphate antibody dilution 1:2000 and anti-mouse-POD labelled antibody dilution 1:500 in incubation buffer are added to each well and the plate is incubated again at RT. The plate is washed again 5 times and 100 µl of TMB-substrate is added. After incubation at RT the reaction is stopped by addition of 100 µl 4 N H_2SO_4 and the plate is read at 450nm/690 nm.

EXAMPLE 2:

Protein tyrosine phosphatase assay

10^6 purified naïve and effector T cells are resuspended in 20 µl of complete medium, activated with soluble anti-CD3, anti-CD45 (each 10 µg/ml), IL-4 (10 ng/ml) and subsequently crosslinked with a F(ab)2 goat anti-mouse IgG antibody (Jackson ImmunoResearch). Cells are snap frozen and prior to the assay lysed in 100 µl of a buffer containing 1% IGEPAL CA-630 (Sigma), 10 mM Tris-HCl pH 7.4; 150 mM NaCl; 400 µM EDTA; 1.4 ml/liter Aprotinin (Bayer) and spun in an Eppendorf centrifuge in order to separate the detergent-insoluble material. 50 µl of the respective supernatant are pipetted into a round-bottom 96-well microtiterplate and 250 µl of assay buffer (100 mM Tric-HCl, pH 7.2; 5 mM EDTA, 10 mM EDTA; 50 mM NaCl; 50 µg BSA/ml; 1.4 ml Trasylol/liter; 5% glycerol) are added. 12 serial 1:2 dilutions are pipetted. In addition, protein concentrations are determined using the BCA assay (Biorad) to ensure the comparability of the samples. p-NPP (Serva) is dissolved in 200 mM Hepes pH 5.6, 200 mM NaCl, 40 mM DTT, 8 mM EDTA at a 40 nM final concentration. 50 µl are pipetted into a flat-bottom microtiter plate and 50 µl of the diluted cell lysate samples are added. After incubation the reaction is stopped by the addition of 100 µl 120 mM NaOH and the absorption is read at 405 nm on a microtiter reader. The peptide TEGQY(PO3H2)QPQP is synthesized and biotinylated, e.g. as appropriate, such as described earlier. 50 µl of a 2 nM solution of this peptide are pipetted into a round-bottom microtiterplate and mixed with 50 µl of the above mentioned cell lysate dilutions. After incubation the reaction is stopped by the addition of 50 µl of 25 mM sodium(ortho)vanadat. 100 µl of this reaction are transferred to a streptavidin-coated plate (StreptaWell High Bind transparent, 96-wells, Roche) and incubated at RT. After several wash steps with

PBS/0.05% Tween 20, 100 µl of antibody mixture consisting of the anti-phosphotyrosine antibody (Sigma) and goat anti-mouse POD antibody (Amersham) is added. After several wash steps, TMB (3,3',5,5'-Tetramethyl-benzidine dihydrochloride, Sigma, T-3405) is added and the reaction stopped by the addition of 100 µl of 4 N sulfuric acid. The enzymatic reaction is read at 450nm/690 nm. OD values are fitted against dilution steps using non-linear regression equations provided by Prism 2.01 (Graphpad Software). To determine the PTPase activity, the dilution step to reach the half maximal OD value is calculated. Significant differences were calculated using the Mann-Whitney U test.

Patent Claims:

1. A method for identifying an agent that modulates the activity of a CD45 tyrosine phosphatase comprising:
a) providing a phosphotyrosine containing peptide, preferably a labeled peptide, e.g. a biotinylated peptide,
b) providing a CD45 tyrosine phosphatase,
c) contacting the peptide of a) with the CD45 tyrosine phosphatase of b) in the absence and in the presence of a candidate compound which is expected to modulate the activity of a CD45 tyrosine phosphatase for a sufficient period of time so that a reaction mixture is formed and reacting for a pre-determined period of time,
d) transferring at least an aliquot of the reaction mixture formed in c) to a solid phase, preferably a solid phase coated with a substrate which is able to bind to the label of the peptide, e.g. a streptavidin-coated solid phase for a biotinylated peptide,
e) detecting the amount of phosphotyrosine moiety of the peptide bound to the solid phase of d) and determining whether there is a difference in the amount of phosphotyrosine in case a candidate compound was present or absent in the reaction mixture, and
f) choosing an agent from said candidate compound detected in e), e.g. for use as a pharmaceutical.

2. A method according to claim 1 wherein the phosphotyrosine containing peptide comprises a sequence selected from the group consisting of T-E-G-Q-Y-Q-P-Q-P, R-N-Q-E-T-Y-E-T-L-K-H and A-E-N-T-I-T-Y-S-L-L-M-H-P wherein Y is phosphorylated.

3. A method according to any one of claims 1 or 2 wherein the phosphotyrosine moiety of the bound peptide is detected with an anti-phosphotyrosine antibody.

4. A kit for identifying an agent that modulates the activity of a CD45 tyrosine phosphatase comprising as components
a) a phosphotyrosine containing peptide, preferably a labeled peptide, e.g. a biotinylated peptide,
b) a CD45 tyrosine phosphatase,
c) an anti-phosphotyrosine antibody, and
d) optionally a solid phase, preferably coated with a substrate which is able to bind to the

label of the peptide.

5. A kit according to claim 4 wherein the peptide comprises a sequence selected from the group consisting of T-E-G-Q-Y-Q-P-Q-P, R-N-Q-E-T-Y-E-T-L-K-H and A-E-N-T-I-T-Y-S-L-L-M-H-P wherein Y is phosphorylated.

6. Use of a peptid comprising a sequence selected from the group consisting of T-E-G-Q-Y-Q-P-Q-P, R-N-Q-E-T-Y-E-T-L-K-H and A-E-N-T-I-T-Y-S-L-L-M-H-P wherein Y is phosphorylated for identifying an agent that modulates the activity of a CD45 tyrosine phophatase.

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Abstract

The invention relates to organic compounds, e.g. to a method for identifying an agent that modulates a CD45 tyrosine phosphatase activity.

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